

PARTIAL CHARACTERIZATION OF THE HEMOLYMPH LIPIDS OF
MERCENARIA MERCENARIA (MOLLUSCA: BIVALVIA) BY
THIN-LAYER CHROMATOGRAPHY AND ANALYSES OF
SERUM FATTY ACIDS DURING STARVATION¹

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Numerous studies have been made on the lipid composition of bivalve molluscs, particularly the sterols, although little information is available on bivalve lipid metabolism (Giese, 1966; Voogt, 1972). It is known that fat digestion in bivalves occurs extracellularly by means of lipases from the crystalline style (George, 1952; Patton and Quinn, 1973). Recently Cheng and Rodrick (1975) demonstrated low lipase activity in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*; and Cheng and Yoshino (1976) examined changes in lipase activity in the hemolymph cells, serum, and mantle fluid of *Mya arenaria* during phagocytosis. Since lipids are digested in the bivalve gut and lipases occur in the hemolymph, knowledge of the lipid content of the hemolymph would greatly aid an understanding of bivalve physiology, specifically the role of the hemolymph in lipid transport.

Lipids are interrelated by their fatty acids; therefore, changes in fatty acid profiles have been used to assess changes in lipid metabolism in a variety of invertebrates (Barrett, 1969; Hoskin, Cheng, and Shapiro, 1974; Pocock, Marsden, and Hamilton, 1971; Schaefer and Washino, 1969). The following investigation was undertaken to determine the neutral lipid composition of the serum and hemolymph cells of *Mercenaria mercenaria* and to examine the variation of serum fatty acid profiles among individual specimens. The reliability of using serum fatty acid profiles to indicate physiological condition resulting from starvation was also investigated.

MATERIALS AND METHODS

Animals

Specimens of the quahog clam, *Mercenaria mercenaria*, from upper Barnegat Bay, New Jersey, were used for analyses of hemolymph neutral lipids. The clams were purchased from a local supplier during the month of July. They were received and bled within 24 hours of digging. Anterior-posterior shell lengths of specimens used were between 7.8 and 8.3 cm. Analyses of serum fatty acids were made on specimens from Buzzards Bay, Massachusetts. They were received within 48 hours after digging. Shell lengths ranged between 8.8 and 10.0 cm. Sex of specimens was not determined. Initial fatty acid analyses were

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made on clams dug in March. Clams used in the starvation study were dug in June.

Hemolymph sampling

Hemolymph was obtained from the sinus of the posterior striated ("quick") adductor muscle using a technique modified after Feng, Feng, Burke, and Khairallah (1971). The shell was notched adjacent to the muscle, a syringe was inserted through the smooth ("catch") muscle into the striated muscle and a 3-4 ml hemolymph sample was slowly withdrawn. The hemolymph samples were immediately centrifuged at 9800 *g*-min to separate the cells from the serum.

For serum fatty acid analyses 1.5 ml hemolymph samples were taken from the specimens on the date of purchase. In the starvation studies specimens were then maintained at 12° C in aerated artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio). The salinity was 29‰. Hemolymph samples were taken after 5 days and 30 days.

To determine whether phytoplankton had grown in the aquarium from cells accidentally introduced with the clams, two 15 ml water samples were collected on the fifth and thirtieth days, centrifuged, and the sediment was examined by light microscopy. No phytoplankton were present, and almost no particulate matter of any kind was observed.

Thin-layer chromatography (TLC)

The lipids were extracted from each hemolymph cell and serum sample according to the procedure of Allen (1972). The extracting solvent consisted of the upper phase of a benzene/isopropanol/water (2/2/1) mixture. Lipases were inactivated by the isopropanol (Gurr and James, 1975).

Analytical grade isopropanol (Fisher Scientific Co., Fair Lawn, New Jersey) and Instra-Analyzed benzene (J. T. Baker Chemical Co., Phillipsburg, New Jersey) were used. All extractions were performed with 2 ml volumes and repeated once for a total of 4 ml. External standards consisting of 3 ml volumes of 0.9% NaCl plus 5 μ l Hormel NLI neutral lipid standard (Hormel Institute, Austin, Minnesota) were employed. These external standards contained 1 μ g each of cholesterol, oleic acid, triolein, methyl oleate and cholesterol oleate. Reagent blank determinations were performed by extracting 3 ml samples of 0.9% NaCl. Glassware was cleaned as previously described (Hoskin *et al.*, 1974).

TLC analyses were performed on 20 \times 20 cm silica gel sheets (Bakertflex IB2, J. T. Baker Chemical Co.). The sheets were predeveloped in chloroform/methanol (2/1) and dried. The sheets were dipped in freshly prepared 8% molybdosilicic acid (Climax Molybdenum Co., New York, New York) in 70% ethanol to about 0.5 cm above the sample application line. All dipped sheets were used within 4 hr.

The samples were applied to the TLC sheets with an automatic spotter (Chromaflex Spotter, Kontes Glass Co., Vineland, New Jersey) connected to a cylinder of dry nitrogen gas. The sample vials were rinsed with 0.2 ml solvent which was similarly applied. Resultant spot diameters ranged between 0.3-0.6 cm. The spots were concentrated into a line at the origin by development in benzene/isopropanol (2/1).

The sheets were developed 12 cm in filter paper lined TLC tanks equilibrated 30–40 min with petroleum ether/diethyl ether/acetic acid (85/15/1). The sheets were dried quickly with a hair dryer, then placed in a 100° C chromatography oven for 5 min. They were scanned within 2 hr with a densitometer (Chromaflex K495000, Kontes Glass Co.) connected to a 10 mV, 10 inch recorder (Model 1005, Beckman-Instruments Inc., Fullerton, California). Peak areas were determined by the method of height times width at half height (Johnson and Stocks, 1971). Peak areas of cell and serum lipid components were compared with peak areas from known amounts of Hormel NLI standard. One lane of every TLC sheet was reserved for a 5 μ l standard and a second lane for a reagent blank or external standard.

Serum cholesterol and triglyceride

Serum cholesterol and triglyceride levels were determined for three samples each consisting of serum pooled from three different specimens. Measurement of cholesterol oxidase activity provided values for total free and esterified cholesterol (Allain, Poon, Chan, Richmond, and Fu, 1974). A commercially available kit was adapted for these analyses (Cholesterol CHOD-PAP, Boehringer-Mannheim, Indianapolis, Indiana).

Triglyceride determinations were based upon total glycerol using a commercially available kit (Eskalab Reagent for Triglycerides, Smith Kline Instruments, Inc., Sunnyvale, California). Additional determinations were performed with an Automatic Clinical Analyser (DuPont Co. Instrument Products, Wilmington, Delaware).

Gas-liquid chromatography (GLC)

A chromatograph with on-column injection and dual flame ionization detectors was used (Model 3920, Perkin-Elmer Corp., Norwalk, Connecticut). Helium carrier gas flow rate was adjusted to 20 ml/min.

To determine the relative amount of cholesterol in the sterol fractions, blood was collected from two specimens, and the lipids from the sera and cells subjected to TLC as described above. Silica gel G on glass plates (Analtech Inc., Newark, New Jersey) was used because of the ease of removing the adsorbant following development. The gel containing sterol was scraped into glass vials, and the sterols were eluted with three washings of benzene, concentrated, and analyzed. Columns were 6 ft. stainless steel, 1/8 inch O.D., packed with 3% SP2250 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania). Cholesterol (Nutritional Biochemicals Co., Cleveland, Ohio) and a cholestane-cholesterol blend (Supelco, Inc.) were used as standards. The cholesterol peak was identified by comparison of absolute retention times of sample peaks with the standards.

Fatty acid methyl esters were prepared directly from 1 ml serum samples by a modification of the procedure of Ferguson (1975). Saponification was accomplished by adding 1 ml of 0.1 N KOH in methanol to each serum sample. The sample tubes were sealed and heated to 100° C in a water bath for 10 min. After the samples cooled, 1 ml of 14% methanolic boron trifluoride was added, the tubes were heated at 100° C for 10 min, cooled, and the fatty acid methyl esters were

extracted with three 1 ml volumes of hexane. One ml amounts of sterile 0.9% saline were identically treated to serve as controls.

The samples were concentrated to dryness under a stream of nitrogen gas, redissolved in 20 μ l hexane, and analyzed. Columns were 6 ft. stainless steel, 1/8 inch O.D., packed with 10% SP216PS on 100/120 Supelcoport (Supelco, Inc.). Column temperature was 180° C. Tentative identification of peaks was made by comparison of absolute retention times with methyl esters of: C_{10:0}, C_{12:0}, C_{13:0}, C_{14:0}, C_{15:0}, C_{16:0}, C_{16:1}, C_{17:0}, C_{18:0}, C_{18:1}, C_{18:2}, C_{19:0}, C_{20:0}, C_{20:1}, C_{20:2}, C_{20:3}, C_{20:5}, C_{21:0}, C_{22:0}, C_{22:1}, C_{22:5}, C_{22:6}, C_{24:1} (Supelco, Inc. or Applied Science Laboratories, Inc., State College, Pennsylvania). Peaks which did not correspond identically with any of the available standards were identified by use of a nomogram constructed from retention time data of the 23 standards according to the method of Haken and Souter (1966).

RESULTS

TLC analysis of neutral lipids

Retardation factors, (R_f) for the standards were: cholesterol, 0.09; oleic acid, 0.24; trioleate, 0.52; methyl oleate, 0.70; and cholesterol oleate, 0.88.

Results from the quantitative determination of twelve 5 μ g standards applied to 10 TLC sheets indicate that the overall coefficient of variation (CV) was between 7 and 12% (Table I). The efficiency of extraction of the 5 μ g external standards from saline was within this experimental variation, so individual corrections for peak areas were not made. Sterols, nonesterified fatty acids, and sterol esters were present in both serum and cells (Table II). Triglycerides were prominent in cell extracts but were present only in trace amounts in serum samples. Fatty acid methyl esters were never detected in cells or serum. An unidentified lipid component with a R_f value of 0.82 was resolved in the blood cell lipid samples. This component was excluded from the value for total cell lipid.

Enzymatic determination of serum cholesterol and triglyceride

Total cholesterol levels for three pooled samples were 5.2 ± 0.4 mg/100 ml, 6.9 ± 0.7 mg/100 ml, and 7.5 ± 0.3 mg/100 ml.

Serum triglycerides were at the 1.0 mg/100 ml level of detection for the serum triglyceride analysis kit and below the level of detection of the automated pro-

TABLE I

Results of twelve 5 μ l amounts of standard applied to ten TLC sheets, separated, then measured by densitometry. Each neutral lipid component is 1 μ g.

Component	Peak areas (mm ²) from densitometer tracings	Coefficient of variation
Cholesterol	160.0 \pm 19.9	12.4%
Oleic acid	350.0 \pm 23.7	6.8%
Triolein	466.8 \pm 58.5	12.5%
Methyl oleate	520.5 \pm 39.7	7.6%
Cholesterol oleate	643.1 \pm 64.9	10.1%

TABLE II

TLC analyses of the neutral lipid composition of hemolymph from twelve specimens of *Mercenaria mercenaria*.

Hemolymph fraction	Sterols	Free fatty acids	Triglycerides	Sterol esters	Per cent total hemolymph lipids (cells plus serum)
Total cells/ml	76.7 ± 17.2%	5.0 ± 3.1%	8.3 ± 3.6%	10.0 ± 5.0%	60.7%
Serum (1 ml)	80.7 ± 24.0%	6.4 ± 3.0%	2.1 ± 1.3%	10.7 ± 9.0%	39.3%

cedure. Duplicate determinations of the three pooled samples yielded values of about 1.0 mg/100 ml. The values for total serum lipids were calculated from the per cent composition of total cholesterol as established by TLC and GLC. The total serum lipids were 44–64 mg/100 ml. Triglyceride comprised 2.1% of the total serum lipid or 0.9–1.3 mg/100 ml. Total cell lipid was 64–95 mg/cells/100 ml, of which triglyceride accounted for 5.3–7.9 mg/cells/100 ml.

GLC analyses of sterols

Eighteen components were detected in each of the serum sterol samples. Cholesterol, the second most abundant sterol, comprised approximately 13% of the total serum sterol fractions. Two components, including cholesterol, accounted for 30 and 35% of the total serum sterols in the two samples.

Sixteen components were resolved in the sterol class from the blood cells. Cholesterol was the most abundant single component in one sample, the second most abundant component in the other sample and accounted for 18% of the total in both samples.

GLC analyses of fatty acids

Twenty-two fatty acid methyl ester peaks were present in chromatograms of the eight specimens examined for serum total fatty acids. Five fatty acids ($C_{16:0}$, $C_{18:1}$, $C_{20:1}$, $C_{20:5}$, and $C_{22:6}$) accounted for 65% of the total serum fatty acids, and eleven fatty acids accounted for over 90% of the total.

Following Deans' (1968) recommendation, the per cent composition was recalculated based only on the major components (Table III). Fourteen fatty acids each had a retention time identical with a standard, the eight other fatty acids were identified by use of the nomogram.

The per cent composition of serum fatty acids was initially more varied in the clams used in the starvation study as shown by the larger standard deviations (Table IV). Since $C_{22:6}$ was not included the relative amounts of the individual fatty acids are proportionately greater than those in Table III. After five days of starvation standard deviations had increased for seven fatty acids when the pooled data were compared. Thus, the amounts of these fatty acids became more varied among the individual specimens as the experiment progressed.

The trends observed were an increase in the percentage of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$. The percentage of $C_{20:1}$ and $C_{20:5}$ decreased (Table II). After five

TABLE III
Serum fatty acids from eight specimens of Mercenaria mercenaria.

Peak number	Probable identity	Per cent composition among the 11 major acids
1	C _{12:1} +	—
2	C _{14:0} *	—
3	C _{14:2} +	—
4	C _{16:0} *	16.8 ± 1.9
5	C _{16:1} *	3.7 ± 0.9
6	C _{17:0} *	—
7	C _{17:1} +	4.1 ± 0.7
8	C _{18:0} *	5.4 ± 1.2
9	C _{18:1} *	10.6 ± 1.4
10	C _{18:2} *	—
11	C _{20:0} *	—
12	C _{20:1} *	9.6 ± 1.5
13	C _{20:2} + C _{21:0} *	3.5 ± 1.0
14	C _{21:1} +	—
15	C _{22:0} *	5.4 ± 0.8
16	C _{20:4} +	4.8 ± 0.5
17	C _{20:5} *	19.9 ± 1.5
18	C _{23:1} +	—
19	C _{24:0} +	—
20	C _{24:1} + C _{22:2} +	—
21	C _{22:5} *	—
22	C _{22:6} *	16.0 ± 1.9

* = identity based on retention time identical to standard.

+ = identity predicted from nomogram.

days of starvation only the change in C_{18:0} met the criterion for statistical significance at the $\alpha = 0.05$ level of the Student's *t*-test. However, all of these changes were significant at the $\alpha = 0.05$ level following 30 days of starvation.

DISCUSSION

Both TLC and enzymatic procedures for determination of serum triglyceride indicated that this class is virtually absent, although TLC revealed that triglycerides were prominent in hemolymph cells. Since triglyceride is practically absent from the serum, lipid is transported primarily as sterol esters, nonesterified fatty acids or as triglyceride within hemolymph cells, rather than as serum triglyceride.

Bayne (1973) reported total lipid levels between 20 and 80 mg/100 ml in pericardial fluid of *Mytilus edulis*. Our values for total serum neutral lipid, based upon total cholesterol and relative per cent composition of the other classes, are between 46 and 65 mg/100 ml.

The sterol composition of molluscs is complex (Patterson, Khalil, and Idler, 1975). We have not attempted to resolve and identify all the hemolymph sterols but have considered only cholesterol, since cholesterol is the major sterol of many *Gastropoda* and *Bivalvia* (Idler and Wiseman, 1971). Our GLC analyses of the relative amount of cholesterol in the sterol classes of the serum and hemolymph

cell fractions have revealed that cholesterol is one of the most abundant, but not the single most abundant component. However, the entire sterol class accounts for the major portion of the total serum lipid.

The four major serum fatty acids, in order of decreasing abundance, were C_{20:5}, C_{16:0}, C_{22:6} and C_{18:1}. These were also the four most abundant fatty acids in the tissues of several other marine bivalves (Kochi, 1975) and in *Mesodesma mactroides*, the yellow clam (de Moreno, Moreno, and Brenner, 1976).

Jefferies (1972) found that C_{16:0}, C_{20:5}, and C_{22:6} were the three fatty acids in greatest amount in whole *Mercenaria mercenaria*. However, C_{18:1} was not one of the four major fatty acids and, in fact, was about equal in amount to C_{18:0}. He found the fourth most abundant tissue fatty acid was C_{20:1}, whereas this was the fifth major serum fatty acid in specimens examined in this study.

In *M. mactroides*, the amount of C_{18:1} showed a seasonal decrease which de Moreno *et al.* (1976) attributed to differences in the available phytoplankton food. Jefferies (1972) also found seasonal differences in two tissue fatty acids of *M. mercenaria*, C_{20:5} and C_{20:4}.

The serum fatty acid profiles of specimens of *M. mercenaria* were remarkably consistent in the clams received in March but less consistent in those received in June. Whether the greater standard deviations of amounts of serum fatty acids in clams dug in June resulted from seasonal or other factors was not apparent.

Farrington, Quinn, and Davis (1973) reported differences in fatty acid composition of specific lipid classes of the polychaete, *Nephtys incisa*, and the bivalve *Yoldia limatula*. They related these differences to pollution levels at the collecting sites. Jefferies (1972) included deviations in fatty acid profiles as part of a pollution induced "stress syndrome" in *M. mercenaria*. Although the basis for the effect of pollution on the fatty acid compositions of these organisms is not known, both pollution stress and starvation resulted in decreased C_{20:5} in *M. mercenaria*.

Analyses of serum fatty acid profiles are attractive because they do not require destruction of the specimen tested. However, in this study they did not provide reliable evidence for the physiological condition of *M. mercenaria* during the first

TABLE IV

Per cent composition of ten serum fatty acids from five specimens of *M. mercenaria* before and after starvation in artificial sea water.

Peak number and tentative identity	Day 0	Day 5	Day 30
4 C _{16:0}	19.4 ± 3.5	22.6 ± 4.9	28.7 ± 6.7*
5 C _{16:1}	4.1 ± 1.0	4.9 ± 1.7	6.0 ± 1.7*
7 C _{17:1}	4.7 ± 1.2	3.7 ± 0.9	2.9 ± 1.7
8 C _{18:0}	6.0 ± 1.2	9.9 ± 1.1*	18.1 ± 3.9*
9 C _{18:1}	13.4 ± 1.8	18.2 ± 4.0	23.8 ± 6.1*
12 C _{20:1}	11.2 ± 2.2	10.9 ± 1.6	7.5 ± 1.2*
13 C _{20:2} + C _{21:0}	4.4 ± 0.7	2.0 ± 1.1	6.2 ± 1.6
15 C _{22:0}	6.1 ± 0.8	6.5 ± 1.6	4.4 ± 1.2
16 C _{20:4}	5.9 ± 0.6	6.0 ± 5.6	7.6 ± 5.5
17 C _{20:5}	24.9 ± 2.1	15.2 ± 6.8	4.7 ± 2.1*

* Mean different at $\alpha = 0.05$ level from the mean on day 0.

five days of starvation. After 30 days of starvation, relative amounts of six fatty acids were statistically different from the initial levels.

SUMMARY

TLC analysis of the neutral lipids in the hemolymph cells and serum of twelve specimens of *Mercenaria mercenaria* was performed. The serum fractions contained sterols (81%), free fatty acids (6%), sterol esters (11%), and a small amount of triglyceride (2%). Absolute amounts of cholesterol and triglyceride were 5.2–7.5 mg/100 ml and about 1 mg/100 ml, respectively. More total lipid and nearly all triglyceride was found to be in the cell fraction of the hemolymph. Cholesterol comprised 18% of the hemolymph cell sterols and 13% of the serum sterols.

The five major fatty acids, in order of decreasing abundance, in the sera of specimens of *Mercenaria mercenaria* harvested in March and June from Buzzards Bay, Massachusetts, were C_{20:5}, C_{16:0}, C_{22:6}, C_{18:1}, and C_{20:1}. A total of 22 fatty acids were resolved with tentative identification made for all of them. Standard deviations of the per cent composition of the major fatty acids were greater among specimens harvested in June than among those harvested in March.

The effect of starvation on the serum fatty acid profile of clams collected in June was determined. Starved specimens had relatively greater amounts of C_{16:0}, C_{16:1}, C_{18:0}, and C_{18:1} and reduced levels of C_{20:1} and C_{20:5}. The C_{20:5} was 20% lower on day 30 than day 0.

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